B8

PCT

INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

(54) Title: METHOD FOR THE DIAGNOSIS OF BLOOD COAGULATION DISORDERS

(57) Abstract

The invention concerns an *in vitro* method that is particularly useful for the diagnosis of thromboembolic diseases, such as hereditary or non-heriditary thrombophilia, and to determine the risk for trombosis in connection with pregnancy, taking anti-conception pills, surgery etc. The method is characterized in thàt the coagulation system in a sample is activated wholly or partly in a manner known per se and incubated with activated Protein C, whereupon a substrate conversion reaction rate like clotting or conversion of a chromogenic substrate is determined. The conversion rate obtained is compared with values obtained for normal plasma samples. If the rate is enhanced, it indicates that the individual from which the sample is derived may suffer from a thromboembolic disease.

1

METHOD FOR THE DIAGNOSIS OF BLOOD COAGULATION DISORDERS.

The present invention concerns a novel method
appropriate for screening and diagnosis of thromboembolic
diseases, e.g. hereditary thrombophilia. The invention can
5   also be used for determining the risk for thrombosis in
pregnant individuals, individuals undergoing surgery,
individuals taking anti-conception drugs etc.

The blood coagulation comprises a complex system of
inter-linked proenzymes, enzymes and cofactors performing
10   its role at the surfaces of activated platelets and
endothelial cells. When the system is activated the
ultimate result is the formation of a blood clot containing
insoluble fibrin. The initiation and termination of fibrin
formation is carefully regulated in normal homeostasis. In
15   vitro, platelets and endothelial cell surfaces are usually
substituted with suitable phospholipids. For the invention
the relevant part of the coagulation system is:
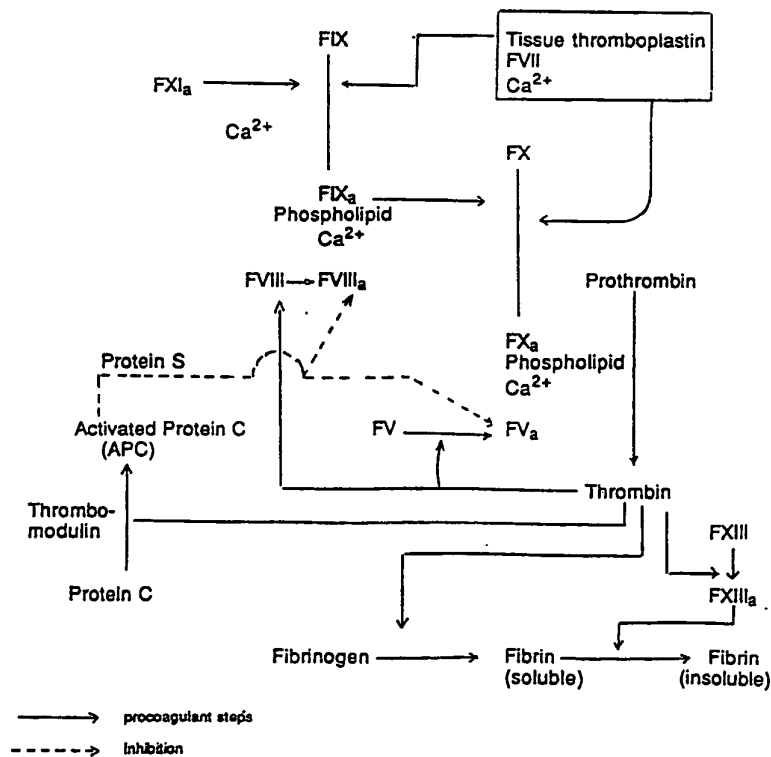
The important part for the invention relates to Protein
C (= PC) and effects produced by action of activated
Protein C (= APC) on the coagulation system. Protein C is a
zymogen that in vitro may be activated by thrombin (=

5 Factor II$_a$) alone, the combination thrombin-thrombomodulin,
certain snake venoms, such as from Akistrodon Contortrix
Contortrix, or purified Factor X$_a$. Activation of sample
endogenous Protein C or addition of exogenously activated
Protein C to a plasma sample will neutralise Factors V$_a$ and

10 VIII$_a$ (degradation) and lead to prolonged times for blood
coagulation in plasma samples of healthy individuals.
Factors V and VIII are activated by small amounts of
thrombin or Factor X$_a$. Protein S is a cofactor to Protein
C.

15 Hereditary heterozygous deficiences in Protein C,
Protein S and Antithrombin III (ATIII, a coagulation factor
opposing coagulation) are found in approximately 10-15% of
patients with diagnosed thromboembolic disease before the
age of 40. Homozygous Protein C and S deficiencies are life

20 threatening and affected individuals develop generalized
microvascular thrombosis and purpura fulminance in the
neonatal period.

Deficiencies in Protein C and S and ATIII are measured
by both functional and immunological methods. One way of

25 determining functional Protein C activity involves the
steps: mixing the plasma sample to be tested with an excess
of human plasma deficient in Protein C and addition of a
Protein C activator and monitoring the appropriate
substrate conversion. Highly specific assays have been

30 achieved by using Protein C specific substrates.
Alternatively one has also utilized substrates for enzymes,
e.g. thrombin and Factor X$_a$, which activities are
influenced by activated Protein C activity (i.e. enzyme
activities that are generated or modulated by APC). In

35 certain cases Protein C assays have involved isolation of
the zymogen and a subsequent activation and addition of
substrate to the activated form. Measurement of Protein C
activity in plasma samples has also been suggested to be

performed directly in the sample without addition of plasma
deficient in Protein C. However, such methods will not
discriminate an abnormality related to Protein C as such
from a disorder related to factors interfering with the
5   effects caused by Protein C (WO-A-91/02812).

Addition of activated Protein C to a plasma sample of a
patient and study of the effect produced has been claimed
to discover a defect Factor $VIII_a$ molecule that is not
degraded by activated Protein C (B. Dahlbäck and M
10   Carlsson, Thromb. Haemost. 65, Abstract 39, 658 (1991)).
However, the data given in the present specification
surprisingly indicate that the patient in question could
not carry a defect Factor·$VIII/VIII_a$.

In order to determine Protein S functional activity in a
15   plasma sample the most common methods involve mixing the
plasma sample with activated Protein C, an excess of
Protein S deficient plasma and further reagents necessary
to achieve clotting (Waart et al., Thromb. Res. 48, 427-37
(1987); Suzuki et al., Thromb. Res. 49, 241-51 (1988);
20   Bertina et al., Thromb. Haemost. 53, 268-72 (1985); and
Comp et al., J. Clin. Invest. 74, 2084-88 (1984). It has
also been suggested to measure Protein S by incubating the
plasma sample with Factor IX_a and activated Protein C and
measurement of the clot time or the conversion of a
25   chromogenic thrombin substrate (KabiVitrum AB (S. Rosén)
WO-A-9101382).

The present inventor has realized that there are
hitherto unrecognized thromboembolic disorders that can be
diagnosed by the addition of activated Protein C to a
30   patient sample containing coagulation Factors and
measurement of an enzyme activity that is influenced by the
APC added. The experimental results now presented indicate
that the disorders in question are related to a hitherto
unknown coagulation Factor(s) or unknown interactions of
35   known Factors. The unknown Factor is not Factor V_a or $VIII_a$
that are resistant to degradation by APC, or an inhibitor
of the immunoglobulin-type for APC. The disorders are
neither related to Protein S deficiency. For simplicity

reasons the unknown Factor(s)/interaction(s) will be referred to as one unknown Factor in this text.

The samples assayed are normally plasma samples, but may also be other types of samples containing the coagulation
5   factors in question. The invention will be illustrated in relation to plasma samples.

Accordingly the invention is an **in vitro** method for the diagnosis of a blood coagulation disorder in a human individual or for the determination of the risk for a human
10 individual to acquire said blood coagulation disorder, said disorder possibly not being expressed by (1) Protein S deficiency, and optionally also by (2) forms of either Factor V_a or Factor $VIII_a$ that are resistant to degradation by APC, or by (3) an inhibitor of the immunoglobulin type
15 for APC. The characteristic feature of the method comprises the steps that:

(i)   a plasma sample obtained from the individual is incubated with

(a)   an exogenous Reagent (I) activating at
20   least partially the blood coagulation system of the sample,

and with

(b)   activated exogenous Protein C (APC) or exogenous PC together with exogenous
25   Reagents (II) that transform PC to APC, and

(c)   further components, such as Ca^{2+} salt and phospholipid or tissue thromboplastin, that are necessary for
30   efficient reaction of the activated factors introduced according to step (i:a), and

(d)   if desired, an exogenous substrate for an enzyme which activity is influenced
35   by activated Protein C;

(ii)   a substrate conversion rate is monitored directly for a blood coagulation enzyme which activity is influenced by activated Protein C,

(iii) the conversion rate determined in step (ii) is
        compared with a standard value being obtained
        from steps (i)-(ii) under identical conditions
        for plasma of normal individuals.

5   In case the substrate conversion rate is not normal
compared to the standard, the individual from which the
sample derives is classified as suffering from the disorder
or being at risk for acquiring the disorder. An increased
conversion rate of the sample indicates a thromboembolic

10   disease or a risk for such a disease (with fibrinogen as
the substrate an increased conversion rate means a
shortened clotting time). The significance of a lowered
conversion rate is at the present stage not known (with
fibrinogen as the substrate a lowered conversion rate means

15   a prolonged clotting time). Probably it is not related to
any disease.

       The range of the normal conversion rate may be quite
broad. Hence, it might, as a complement, be of value to run
steps (i)-(ii) on a plasma sample from the individual with

20   exclusion of the incubation according to (i:b) and compare
the result obtained with that obtained according to the
invention.

       The incubation according to (i:a) serves to introduce an
activated coagulation factor that can be used for the

25   measurement in step (ii). The expression "partially" means
that the addition of Reagent (I) leads to the presence of
at least Factor IX_a. Reagent (I) may be a certain
coagulation factor or a reagent that activates the system
via the intrinsic or extrinsic pathway. Accordingly Reagent

30   (I) may be Factor IX_a or Factor XI_a (intrinsic pathway),
Factor XII_a (intrinsic pathway), kallikrein (intrinsic
pathway), a contact activator (intrinsic pathway) such as
kaolin, celite or ellagic acid (intrinsic pathway), an APTT
reagent (Activated Partial Thromboplastine Time; i.e. a

35   reagent containing a phospholipid and a contact activator
(intrinsic pathway)), tissue thromboplastin (PT-reagent, PT
= Prothrombin time, (extrinsic pathway)). In cases where a

poor specificity is acceptable Reagent (I) may also be
Factor X_a.

   Protein C (i:b) may be of various origin. In case the
Protein C and the sample are of different species origin it
5   is highly recommendable to include Protein S (cofactor to
activated Protein C) in the incubation mixture. Protein C
and Protein S should be of the same species origin, for
instance bovine Protein C requires bovine Protein S.
Protein C is preferably activated prior to being added,
10   although activation may also be accomplished after it has
been added to the sample. Activation shall take place under
standardised and definied conditions. Normal activation
agents are those given on page 2. Recombinantly produced
biologically functional forms of Proteins C and S can also
15   be used.

   The components used according to step i:c depend on the
mode employed and may necessitate the inclusion of plasma
protease inhibitors for enzymes other than the monitored
one or of a fibrin polymerization inhibitor. Ca^{2+} may be in
20   the form of a plasma soluble salt that provides the Ca^{2+}
ion in free uncomplexed form, i.e. strong Ca^{2+} chelators
should be avoided. In the final assay medium the
concentration of Ca^{2+} may be selected within 0.5-50 mM,
preferably within 5-15 mM, such as 6-7 mM. Too high a
25   concentration may inhibit the coagulation system.

   The substrate according to (i:d) is normally a
synthetic substrate for an enzyme which activity is
influenced by activated Protein C, e.g. thrombin (=
Factor II_a) and Factor X_a. Suitable synthetic
30   substrates are water soluble and have preferably
oligopeptide structure with three, four or five amino
acid residues and an amino terminal that is protected
from being attacked by amino peptidases. The
protection is accomplished either by a protecting
35   group or by having a D-amino acid in the amino
terminal. In order to give a detectable response the
carboxy terminal of a synthetic substrate is amidated
with a group that specifically can be released and

detected upon action of the relevant blood coagulation
protease. The group to be released is selected among
chromogenic, fluorogenic or chemiluminogenic groups
and other analytically detectable groups. Se further
5   H.C. Hemker, "Handbook of synthetic substrates for the
coagulation and fibrinolytic system", Martinus Nijhoff
Publishers, 1983, and J. Fareed et al, "Synthetic
peptide substrates in hemostatic testing" in CRC
Critical Reviews in Clinical Laboratory Sciencies Vol
10   19, Issue 2, 71-134(1983). In case of samples other
than plasma samples exogenous fibrinogen may be added
as substrate.

The order of addition and the incubation vary with the
mode of the invention. For instance in case Reagent (I) is
15   an APTT reagent (i:a) and the substrate conversion to be
monitored is fibrinogen to fibrin, reagent (I) is added to
the sample and allowed to maximally activate Factor XI to
Factor XI_a. Then Ca^{2+} (i:c) is added and the time for
clotting measured. Activated Protein C according to step
20   (i:b) is introduced either simultaneously with, prior to or
after the activation to Factor XI_a. A PT-assay is performed
similarly with addition of tissue thromboplastin (instead
of the APTT reagent) to the sample in an amount sufficient
for activation of Factor X to Factor X_a or Factor IX to
25   Factor IX_a. Thereafter activated Protein C (i:b) is added
and finally the clotting time is measured as in any APTT
assay. In case a synthetic substrate is used it can be
added at any stage before or at the start of the monitoring
reaction. In order to run the monitoring reaction with high
30   specificity, the above-mentioned inhibitors may be
introduced at any suitable stage into the reaction medium.
For instance it may be appropriate to add a thrombin
inhibitor together with a substrate for Factor X_a, when
Factor X_a activity is measured. The same inhibitor added
35   prior to addition of the substrate may, however, adversely
affect the formation of Factor X_a.

In order to accomplish a specific result with respect to the above-mentioned unknown Factor one should try to keep the patient plasma sample content of the final assay medium as high as possible. Accordingly patient plasma sample

5   content in tests having good specificity should be >10 %, in particular >20% or >35% (v/v).

It may be practical to sell and use reagents according to (i:a-d) in predispensed combinations that may have been lyophilized separately or as mixtures containing at least

10   two of the components given in (i:a-d), preferably in the doses used for testing. It may also be practical to have performed the lyophilization in the vial to be used in the assay. Suitable combinations are (concentration ranges refer to values during the assay, preferred ranges are

15   given within brackets):

A.   <u>APTT based clot methods and APTT depending clot methods for factors V and VIII.</u>

1. Human APC	$10ng/mL-50/ug/mL$
	$(25ng/mL-10/ug/mL)$
2. APC species (non-human)	$100ng/mL-50/ug/mL$
	$(10ng/mL-50/ug/mL)$
3. Bovine APC/Bovine Protein S, from other non-human species.	APC: $5ng/mL-5/ug/mL$ Protein S: $100ng/mL-20/ug/mL$ $(10ng/mL-20/ug/mL)$

All reagents given in 1-3 above and intended to be used in the invention may be lyophilized in the absence or presence of Ca^{2+}. If present, the amount of Ca^{2+} should give a Ca^{2+} concentration of 0.5-30mmol/L

30   in the final assay medium. Phospholipid may be included in the lyophilized preparations.

B.   <u>APTT modified clot methods in which contact factor activation has been excluded.</u>

Factor IX_a	$0.05\ ng/mL-2/ug/mL$
Factor XI_a	$0.05\ ng/mL-2/ug/mL$
Factor XII_a	$0.05\ ng/mL-2/ug/mL$
Kallikrein	$0.05\ ng/mL-2/ug/mL$

No limitation regarding species. FIX_a may also be used together with any of the combinations A 1-3 including the presence or absence of Ca^{2+} and phospholipid.

C.   APTT chromogenic methods.

Combinations according to A 1-3 with inclusion of fibrin polymerization inhibitor (concentration $>/= K_I$, K_I = inhibition constant) and chromogenic substrate (concentration $>/= 0.1K_m$, K_m = Michaelis-Mentens constant). Alternatively the chromogenic substrate is lyophilized separately or in the presence of Ca^{2+} that in turn optionally is combined with a fibrin polymerization inhibitor. In a minor alternative the substrate is lyophilized together with a fibrin polymerization inhibitor but in the absence of Ca^{2+}. The constituents shall provide conditions such that no disturbing substrate hydrolysis takes place during reconstitution.

D.   APTT modified chromogenic methods in which contact factor activation has been excluded.

Combinations of reagents as given under B and C.

E.   PT clot method utilizing tissue thromboplastin.

Reagents according to A 1-3 optionally combined with Ca^{2+} and/or tissue thromboplastin.

F.   Modified clot method for screening of Factor V defect.

Factor X_a 0.02ng/mL-0.5/ug/mL

Factor X_a is not limited to species. The reagent may contain combinations according to A 1-3 optionally together with Ca^{2+} and/or phospholipid.

G.   PT chromogenic method.

Combinations according to C and E above but with thromboplastin instead of phospholipid.

H.   Chromogenic Factor VIII method.

Reagents according to A 1-3 employed in a standard chromogenic Factor VIII assay. The reagents may have been lyophilized together with either of Factor IX_a +/- Ca^{2+} +/- phospholipid or Factor X +/- Ca^{2+} +/- phospholipid, with Factor X concentration of

0.1/ug/mL-50/ug/mL. The reagent may also comprise
inclusion of small amounts of thrombin and, when
Factor X is included, also Factor IX$_a$. Furthermore, a
chromogenic substrate for Factor X$_a$ may be included in
5   the reagent.

I.   Chromogenic Factor V method.
     Reagents according to C and F with or without
     inclusion of prothrombin (0.02ng/mL-50/ug/mL)
APTT reagents may be included in combinations A-D, provided
10 they are not co-lyophilized with Ca^{2+}. Active enzymes and
their substrates may be co-lyophilized as recently
described (EP-A-318,571).

     The invention is primarily intended as a screening
method in order to find individuals that need further
15 diagnostication, but comprises also specific factor assays
according to F, H and I above. The proper selection of
reagent (I) and substrate to be monitored (i:d) refine the
possibility of finding where in the coagulation system a
diagnosed disorder is located. In principle the inventive
20 method will detect disorders related to defective
interactions between activated Protein C and Factor V$_a$,
Factor VIII$_a$. It will also detect the presence of
inhibitors of activated Protein C, and abnormalties in
hitherto unrecognized interactions and factors influenced
25 by Protein C activation or activated Protein C activity.

     The invention will now be illustrated by way of the
inventors discovery of a patient suffering from a novel
disorder in the blood coagulation system. The appending
claims are an integral part of the description.

<u>EXPERIMENTAL PART</u>

<u>Case Report</u>:

    The proband is a male born in 1942. In 1961, he had the
first episode of deep venous thrombosis in one of the legs.
5 After this, he was healthy and free of thrombosis for
almost 20 years. Between 1980 and 1987 he had multiple
thrombotic episodes, occurring at least once a year. The
thrombotic events were treated with vitamin K antagonists
for up to three months. A thrombus was positively verified
10 with flebography at least at two occasions. The proband has
developed a post-thrombotic syndrome in his legs. He has no
other disorders. In 1987, he quit smoking and at the same
time he started taking aspirin daily. During 1987-1991 he
has not experienced any thrombo-embolic episode. Both male
15 and female members of the patient's family have similar
histories with multiple episodes of deep venous thrombosis.
His 10 year older brother have had deep venous thrombosis
at multiple occasions, most of them occurring between the
age of 45 and 50. The proband also reports that an uncle on
20 his mother's side has had a medical record with multiple
episodes of thrombosis. The patient's mother was born in
1905, and she has had episodes when deep venous thrombosis
has been suspected clinically. Two more brothers and a
sister have had incidences where thrombosis has been
25 suspected.

**Known coagulation and immunological methods used as a
complement to the inventive method for the diagnosis.**

---

30   The activated partial thromboplastin time (APTT, Organon
Technica), Owren's P&P, thrombin time and reptilase time
were determined with standard methods. Antithrombin III was
measured with an amidolytic assay (Coatest ATIII, Kabi
Diagnostica, Molndal, Sweden). Total and free Protein S and
35 Protein C antigen levels were determined with previously
described immunochemical methods (Malm J. et al., Br. J.
Haemat. 68, 437-443 (1988). Protein C function activity was
analysed with a synthetic substrate after activation with
the venom from <u>Agkistrodon Contortrix Contortrix</u> using a

commercially available kit (Coatest Protein C, Kabi
Diagnostica AB, Molndal, Sweden).

Absorption of IgA, IgG and IgM was performed as
previously described (Dahlbäck B. et al., Blood 62, 218-225

5 (1983).

A second functional Protein C assay was also performed
as previously described (Hickton C.M., Thromb. Res. 41 501-
8, (1986)). The method included barium-citrate absorption
of plasma. The proteins that bound to the barium-citrate

10 were eluted and the eluate was incubated with a thrombin-
thrombomodulin complex to activate Protein C. The amount of
APC after activation was quantified using an APTT clotting
assay.

15 **Inventive methods**

1.   An APTT based method was used to determine the
anticoagulant effect of purified APC in patient
plasma. In this method (APC-APTT assay) the APC

20   mediated prolongation of the APT-time was measured as
follows: 0.1 ml plasma was incubated with 0.1 ml APTT
reagent for 5 minutes at 37°C before addition of 0.1
ml of an APC-Ca^{2+} mixture (0-20 /ug/ml APC in 10 mM
Tris-HCl, 0.15 M NaCl, 30 mM calcium chloride pH 7.5

25   containing 0.1 % bovine serum albumin (BSA)) which
initiated blood coagulation. The APC was prepared as
previously described (Dahlbäck B. et al., J. Biol.
Chem. 261, 12022-12027 (1986). The assay was run with
bovine APC with or without the presence of bovine

30   Protein S.

    Normally APTT assays are run without addition of
NaCl. Accordingly, but also because NaCl prolongs the
coagulation times, it is preferred to run this mode of
the invention without addition of NaCl.

35 2.   In order to determine the effect of APC on plasma
Factor V, increasing concentrations of APC (10 /ul
diluted in 10 mM Tris-HCl, 0.15 M NaCl, pH 7.5
containing 0.1 % BSA) were added to 0.09 ml plasma.
Immediately after the APC-addition, 0.1 ml rabbit

brain cephalin (diluted in 0.15 M NaCl) and 0.1 ml 30
mM $CaCl_2$ were added. After incubation for 15 seconds
at 37°C, clotting was initiated with 0.1 ml Factor X_a
(150 ng/ml diluted in 10 mM Tris-HCl, 0.15 M NaCl, pH

5   7.5 containing 0.1 % BSA). In the absence of APC, this
Factor X_a concentration gave an approximate clotting
time of 30 seconds in the control plasma used. Factor
X_a was prepared as described previously (Dahlbäck B.
et al., J. Biol. Chem. 261, 12022-12027 (1986)).

10 3.   A modification of a commercial Factor VIII assay
(Coatest Factor VIII, Kabi Diagnostica AB, Molndal,
Sweden) was used to analyse the effect of APC on
plasma Factor VIII. Patient or control plasma (25 /ul
1/125 to 1/400 in 10 mM Tris-HCl, 0.15 M NaCl, pH 7.5

15   containing 0.1 % BSA) was incubated with 75 /ul of the
kit reagent containing Factor IX_a, Factor X,
phospholipid and Ca^{2+}. Just prior to the test,
increasing concentrations of APC (0.1-100 /ug/ml) were
added to this reagent. After 20 minutes incubation at

20   37°C, 50 /ul of a mixture of the synthetic substrate
(2.7 nM) (Bz-Ile-Glu(gamma-OR)-Gly-Arg-pNA = S-2222,
Kabi Diagnostica, Molndal, Sweden) and the thrombin
inhibitor (60 /uM) (N-dansyl-(p-guanidino)-Phe-
piperidide = I-2581, Kabi Diagnostica AB, Mölndal,

25   Sweden) was added. After incubation for 10 minutes
more at 37°C, the reaction was interrupted by the
addition of 150 /ul (1 M) citric acid and the
absorbance was measured at 405 nm. A standard curve
for Factor VIII was made using normal plasma diluted

30   from 1/100 to 1/800.

Results of the methods used in the cause of the
investigation.

───

35   The APT-time together with values for ATIII and Protein
S were normal and the patient had no indications of the
presence of lupus anticoagulants. The plasma level of
Protein C was normal, both when being measured with the
immunological method and with the functional assay, which

included Protein C activation with snake venom and
quantitation of APC with a synthetic substrate.

    The barium-citrate functional Protein C assay gav
consistently lower Protein C values for the eluate when

5   diluted 1:10 compared to 1:40. This might indicate the
presence of an inhibitor for APC. In order to check this,
we used the inventive APC-APTT assay. The clotting time
obtained was always shorter than for the control plasma. In
order to rule out an inhibitor of the immunoglobulin type,

10   the patient's plasma was depleted completely in IgA, IgG or
IgM by absorption. The shortened clotting time did not
disappear. The results found could be due to a functional
Protein S deficiency. However, this possibility was ruled
out since bovine APC, when added with or without bovine

15   Protein S is considerably less efficient in prolonging the
APT-time of the proband's plasma compared to prolongation
in the control plasma.

    A third possible mechanism for the observed APC-
resistance was that the proband's Factor V_a or Factor $VIII_a$

20   could be resistant to cleavage by APC. To elucidate this
possibility, assays were devised which directly measured
the inhibition of plasma factors V_a and $VIII_a$ by APC. Using
the Factor X_a based clotting assay (described above), the
inhibition of patient Factor V_a by APC was found to be

25   normal suggesting that Factor V_a in the patient's plasma
was degraded in a normal fashion by exogenously added APC.
This experiment ruled out the possibility of a Protein C
inhibitory antibody explaining the APC-resistance. To test
the remaining possibility, i.e. that APC could not degrade

30   the proband's Factor $VIII_a$, the effect of added APC in a
Factor $VIII_a$ assay was tested. However, the proband's
Factor $VIII_a$ was found to be normally degraded by the added
APC when compared to control plasma. This finding is
contrary to the inventor's earlier publication (B. Dahlbäck

35   and M Carlsson, Thromb. Haemost. 65, Abstract 39, 658
(1991)).

    To investigate whether the APC-effect was inherited, 18
family members were analyzed using the APC-APTT assay. 10

(both male and female) of the 18 tested family members did not respond to APC with normal prolongation of their clotting times, which suggests that the factor molecule responsible for the effect is resistant to APC. This result
5   shows that the defect molecule was inherited and present in the family members that did not give normal prolongation of their clotting times. It is noteworthy that in the absence of added APC, the APT-times of these individuals and of the proband were shorter than for the controls. This may
10   suggest partial degradation of relevant Factor molecules during APTT assays of normal plasma. To test the sensitivity of the APC-APTT assay for the presence of APC-resistance, mixtures of proband and normal plasma (1:1, 1:10 and 1:100) were analyzed. When added to the 1:1
15   mixture, APC was equally inefficient in prolonging the clotting time as when added to the proband's plasma. Half of the normal prolongation was observed when testing the 1:10 mixture, whereas the 1:100 mixture behaved like control plasma. Thus the APC-APTT assay did not
20   discriminate between the presence of 50 % and 100 % APC resistant factor molecules suggesting the method to be a useful screening method for the identification of carrier states.

      Since the measured lack of substantial prolongation of
25   the clotting time is not related to Factors $VIII_a$ and V_a or a Protein C inhibitor of the immunoglobulin type or a defective Protein S - APC interaction the effect is likely to be associated with a hitherto unrecognized coagulation factor.
30   The APC-APTT assay according to the invention has also been run on plasma samples from about 100 patients with diagnosed thrombosis. About 10% of the patients gave shortening of their clotting times compared to the standard. No apparent hereditary could be seen. None of the
35   patients had been found positive in other assays for the determination of coagulation disorders.

      An APC-APTT method similar to method 1 under the heading Inventive Methods 1 given above has been run on plasma

samples from Protein S deficient patients. In this specific
mode the APC amount was adjusted so that pooled normal
plasmas resulted in a prolongation of the coagulation time
of 40 seconds. The new type of patients we have detected

5   then gave a prolongation of coagulation time of 0-15
seconds while plasma from patients with diagnosed Protein S
deficiency gave a prolongation time that was close to
normal. In order to check further the influence of Protein
S deficiency we also assayed normal plasma that had been

10 made deficient in Protein S by immune adsorption. The
prolongation time decreased with about 50% which indicates
that the prolongation measured for our new patient group is
not caused by Protein S deficiency. We have also added
Protein S to plasma from the new patient group and run the

15 inventive method on such plasma. The result has been that
the prolongation of the coagulatiuon time is not normalised
which further supports that the inventive method does not
measure Protein S.

By varying the plasma content of the assay medium it was
20 experimentally verified that one should avoid too low
plasma concentrations in the final assay medium.

**Restriction digestion of DNA, PCR and hybridization for
assaying a Factor VIII gene X-linked inheritance of the**
25 **disorder.**

DNA from three relatives (the proband, his mother and
one of his brothers) that were suspected of carrying a gene
for the disorder was subjected to PCR with amplification of
30 the Factor VIII gene and subsequent cleavage with Bcl 1 as
described previously (Kogan et al., N. Engl. J. Med. 317,
985-90 (1987)).

In the human population this treatment leads to two
different fragments (142 kb and 91 kb, respectively). The
DNA of an individual will carry genes giving either both
fragments or only one fragments. The mother's DNA gave both
5   142 kb and 91 kb fragments, while one of her sons gave only
the 142 kb fragment and the other only the 91 kb fragment.
This is a clear indication that the two sons have received
different Factor VIII genes from their mother. The disorder
traced could thus not be linked to a gene on an X-
10   chromosome.

PATENT CLAIMS

1. An in vitro method for the diagnosis of blood
   coagulation disorders, in particular thromboembolic
   diseases, in a human or for the determination of the
   risk for a human to acquire blood coagulation
   disorders, wherein said disorders are not expressed by
   Protein S deficiency or defective Factor VIII$_a$,
   comprising

   (i)   incubating a sample containing human
         coagulation factors with

         (a)   an exogenous Reagent (I) which at least
               partially activates the blood coagulation
               system of the sample,

         (b)   an activated exogenous Protein C (APC),
               or exogenous PC together with exogenous
               reagents (II) that transform exogenous PC
               to APC, and with

         (c)   components, such as phospholipid and Ca^{2+}
               salt, that are necessary for efficient
               reaction of the activated coagulation
               factors introduced by step (i:a), and,

         (d)   if desired, an exogenous substrate for an
               enzyme, which activity is influenced by
               activated Protein C;

         the final assay media preferably having at
         least a patient plasma sample content that is
         >10 %, in particular >20% or >35% (v/v).

   (ii) directly monitoring a substrate conversion rate
         for a blood coagulation enzyme which activity is
         influenced by activated Protein C; and

   (iii) comparing the conversion rate determined in step
         (ii) with a standard value obtained from samples
         of normal individuals subjected to steps (i) and
         (ii) under identical conditions;

   wherein the finding of a sample conversion rate that
   is not normal compared to the standard value is taken
   as an indication of the human suffering from the
   disorder or as being at risk for acquiring the

disorder, in particular an enhanced conversion rate is taken as an indication of a thromboembolic disease or a risk for acquiring such a disease.

5   2.   An in vitro method for the diagnosis of blood coagulation disorders, in particular thromboembolic diseases, in a human or for the determination of the risk for a human to acquire blood coagulation disorders, said disorder not being related to Protein

10   S deficiency, comprising

(i)   incubating a sample containing human coagulation factors with

(a)   an exogenous Reagent (I) which at least

15   partially activates the blood coagulation system of the sample,

(b)   an activated exogenous Protein C (APC), or exogenous PC and exogenous reagents (II) that transform exogenous PC, and

20   optionally also endogenous PC, to APC, and with

(c)   components, such as phospholipid and a Ca^{2+} salt, that are necessary for efficient reaction of the activated

25   factors introduced by step (i:a), and,

(d)   if desired, an exogenous substrate for an enzyme, which activity is influenced by activated Protein C;

the final assay media preferably having at

30   least a patient plasma sample content that is >10 %, in particular >20% or >35% (v/v).

(ii)   directly monitoring a substrate conversion rate for a blood coagulation enzyme which activity is influenced by activated Protein C; and

35   (iii) comparing the conversion rate determined in step (ii) with a standard value obtained for samples of normal individuals subjected to steps (i) and (ii) under identical conditions;

wherein the finding of a sample conversion rate that
is not normal compared to the standard value is taken
as an indication of the human suffering from the
disorder or as being at risk for acquiring the
disorder, in particular an enhanced conversion rate is
taken as an indication of a thromboembolic disease or
a risk for acquiring such a disease.

3. The method of claim 1 or claim 2, **characterized** in that
Reagent (I) is
(a)   the components necessary to activate the
blood coagulation system of the sample via
the intrinsic pathway, such as an APTT
reagent, a contact activator, Factor IX_a,
Factor XI_a, Factor XII_a and kallikrein,
and/or
(b)   the components necessary to activate the
blood coagulation system via the extrinsic
pathway, e.g tissue thromboplastin,
and the components according to (i:b-d) are added
simultaneously with or, where appropriate, after
Reagent (I) has been allowed to incubate with the
sample for a sufficient time to activate the intrinsic
respective extrinsic pathway.

4. A method according to claim 1 or claim 2, wherein
Reagent (I) is exogenous Factor X_a or exogenous Factor
IX_a, said incubation optionally being performed in the
presence of exogenous prothrombin.

5. A method according to claim 1 or claim 2, wherein
Reagent (I) is exogenous Factor X in excess of sample
endogenous Factor X and in combination with exogenous
Factor IX_a, possibly together with thrombin.

6. A method according to claim 3, wherein Reagent (I) is
an APTT reagent.

7. A method according to any of claims 1-6, wherein the
    monitored substrate conversion is conversion of the
    endogenous fibrinogen of the sample to fibrin which is
    monitored via clot formation, with exogenous substrate
5   not being present.

8. A method according to any of claims 1-7, wherein
    exogenous substrate is present and its conversion is
    monitored, if necessary together with a fibrin
10 polymerization inhibitor.

9. A method according to claim 8, wherein the exogenous
    substrate is specific for Factor X_a and is present
    together with a fibrin polymerization inhibitor.

15
10. A method according to claim 8, wherein the exogenous
    substrate is specific for thrombin and a fibrin
    polymerisation inhibitor is present.

20 11. A method according to any of claims 1-6 and 8-10,
    wherein a fibrin polymerization inhibitor together
    with the exogenous substrate are present during the
    incubation and that the conversion of the exogenous
    substrate is monitored, said exogenous substrate being
25 a synthetic peptide that preferably consists of less
    than five amino acid residues and a group that is
    specifically released and becomes analytically
    detectable upon action of the enzyme, e.g upon action
    of thrombin or Factor X_a, said group being bound in an
30 amide linkage to the carboxy terminal of the peptide
    and being selected from chromogenic, fluorogenic and
    chemiluminogenic groups (= chromogenic substrate).

12. A method according to any of claims 1-11, wherein
35 Protein C is human Protein C or non-human Protein C
    (such as bovine Protein C), possibly together with
    Protein S from the same species as Protein C.

13. A method according to any of claims 1-12, wherein at
    least one of the materials of (a), (b), (c) or (d) is
    in lyophilized form that is reconstituted in
    connection with the assay.

A. CLASSIFICATION OF SUBJECT MATTER

IPC5: C12Q 1/56, G01N 33/86
According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

IPC5: C12Q G01N

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

SE, DK, FI, NO classes as above

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)

WPI, WPIL, MEDLINE, BIOSIS, EMBASE

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	SE, B, 464135 (KABIVITRUM AB), 11 March 1991 (11.03.91), see especially claim 2, example 6 and page 9 lines 21-36	1-13
A	Thromb. Haemost, 65, Abstract 39, 658, 1991, page 658, Björn Dahlbäck et al: "Factor VIII defect associated with familial thrombophilia".	1-13
A	WO, A1, 9102812 (MICHIGAN STATE UNIVERSITY), 7 March 1991 (07.03.91), see especially claim 1	1-13

[X] Further documents are listed in the continuation of Box C.	[X] See patent family annex.

* Special categories of cited documents:

"A" document defining the general state of the art which is not considered to be of particular relevance

"E" erlier document but published on or after the international filing date

"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance: the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone

"Y" document of particular relevance: the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art

"&" document member of the same patent family

Date of the actual completion of the international search	Date of mailing of the international search report
15 February 1993	2 3 -02- 1993

Name and mailing address of the ISA/	Authorized officer
Swedish Patent Office Box 5055, S-102 42 STOCKHOLM	
Facsimile No. +46 8 666 02 86	Carolina Palmcrantz Telephone No. +46 8 782 25 00

Form PCT/ISA/210 (second sheet) (July 1992)

C (Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT		
Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A	WO, A1, 9101383 (MICHIGAN STATE UNIVERSITY), 7 February 1991 (07.02.91), see especially claims 1 and 4	1-13
A	EP, A1, 0434377 (ORTHO DIAGNOSTIC SYSTEMS), 26 June 1991 (26.06.91), see especially claims 1, 6-7	1-13

International application No.

PCT/SE 92/00310

Patent document cited in search report		Publication date	Patent family member(s)		Publication date
SE-B-	464135	11/03/91	AU-A-	6053390	22/02/91
			CA-A-	2063564	15/01/91
			EP-A-	0486515	27/05/92
WO-A1-	9102812	07/03/91	US-A-	5051357	24/09/91
WO-A1-	9101383	07/02/91	US-A-	5051357	24/09/91
EP-A1-	0434377	26/06/91	US-A-	5169786	08/12/92